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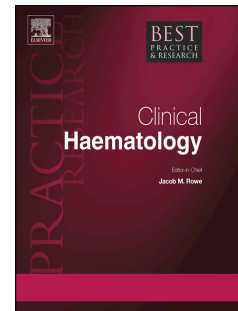
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Pre-clinical development of Chimeric Antigen Receptor T-cell immunotherapy:
implications of design for efficacy and safety

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**Pre-clinical development of Chimeric Antigen Receptor T-cell immunotherapy:
implications of design for efficacy and safety**

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Abstract

Following the landmark approvals by the United States Food and Drug Administration, the adoptive transfer of CD19-directed chimeric antigen receptor (CAR) T-cells has now entered mainstream clinical practice for patients with chemotherapy-resistant or refractory B-cell malignancies. These approvals have followed on from a prolonged period of pre-clinical evaluation, informing the design of clinical trials that have demonstrated unprecedented efficacy in this difficult to treat patient population. However, the delivery of autologous CAR-engineered T-cell therapy is complex, costly and not without significant risk. Here we summarize the key themes of CAR T-cell preclinical development and highlight a number of innovative strategies designed to further address toxicity and improve efficacy. In concert with the emerging promise of precision genome editing, it is hoped these next generation products will increase the repertoire of clinical applications of CAR T-cell therapy in malignant and perhaps other disease settings.

Key words

Adoptive cell therapy • chimeric antigen receptor • engineering • cancer immunotherapy

Introduction

Chimeric Antigen Receptor (CAR) T-cell immunotherapy has shown substantial anti-tumor activity against refractory B-cell malignancy [1]. Indeed, therapeutic efficacy is unprecedented for a new cancer medicine with response rates of up to 90% for patients with relapsed/ refractory B-cell acute lymphoblastic leukemia (B-ALL) and over 60% for patients with non-Hodgkin's lymphoma (NHL) [2]. Immunotherapy using CAR T-cells is generally undertaken following the administration of lymphodepleting chemotherapy. This intervention

increases access to supportive cytokines (e.g. IL-7 and IL-15) [3], creates a viable spatial compartment for CAR T-cell growth and persistence [4] and depletes systemic and tumor-resident CD4⁺ CD25⁺ regulatory T-cells (Tregs) [5]. Nevertheless, toxicity of CAR T-cell immunotherapy administered in the context of prior lymphodepletion can be life threatening, a factor that requires careful consideration during pre-clinical development.

Structure of Chimeric Antigen Receptors

Chimeric antigen receptors are engineered proteins that contain an antigen recognition domain - most commonly a single chain variable fragment (scFv) - linked via a hinge or spacer and transmembrane domain to a bespoke signaling domain [6] (Figure 1). In initial configurations, a T-cell receptor (TCR)-like signal 1 alone was provided, generally using a module that contains one or more immunoreceptor tyrosine-based activation motifs (ITAM), such as CD3 ζ . However, immunotherapy using T-cells that were engineered to express these “first generation” CARs proved ineffective, largely owing to inadequate T-cell persistence and expansion *in vivo* [7]. Second and third generation CARs respectively incorporate one or two intracellular co-stimulatory domains such as CD28, CD137 (4-1BB), OX40 or ICOS, thereby providing signal 2 for T-cell activation [7]. This development translated into improved CAR efficacy and functionality, although it remains doubtful as to whether third generation CARs are truly superior to their second generation counterparts. More recently, a range of fourth generation CARs have been described in which additional signals are delivered to enhance potency (e.g. inducible release of interleukin (IL)-12). Since CAR T-cells bind in an antibody-like manner, targeting is independent of human leukocyte antigen (HLA) haplotype or tumor-associated HLA downregulation. Furthermore, engagement of non-protein antigens such as carbohydrates and gangliosides can be achieved.

Selection of scFv for CAR T-cell targeting

Target selection is crucial in the design of CARs to achieve optimal safety and efficacy [8]. Success of CAR T-cell immunotherapy of B-cell malignancy reflects the widespread expression of CD19 on the transformed cell population, but not on hematopoietic stem cells or terminally differentiated plasma cells. Building on this, CARs targeting other B-cell antigens (e.g. CD20 and CD22) for the treatment of leukaemia and lymphomas have been developed. This is especially important as loss of CD19 expression has been reported in relapsed patients and CD19 negative populations have been found in malignancies.

Almost all anti-CD19 CARs currently undergoing clinical evaluation contain murine scFvs. While durable remissions have been achieved, CD19⁺ disease relapse may also ensue, accompanied by the loss of circulating CAR T-cells [9]. While this may be influenced by choice of co-stimulatory domain [10] and tonic signaling [11], T-cell responses against epitopes derived from murine scFvs may occur. This highlights the desirability of selection of humanized and fully human scFvs, as recently evaluated in patients with advanced NHL [12]. A novel approach to enhance target antigen repertoire involves the re-direction CAR specificity against HLA-restricted peptide antigens that are derived from intracellular tumor antigens, such as Wilms Tumour (WT)1. Use of scFvs that bind specifically to individual HLA/peptide complexes significantly expands the range of potential CAR targets and holds great potential to expand the successful and safe delivery of CAR T-cell immunotherapy [13].

Selection of hinge/spacer and transmembrane elements

Selection of hinge or transmembrane region can also influence activity of CAR T-cell immunotherapy. Illustrating this, second generation CD19-specific CAR T-cells that contain a CD28 hinge and transmembrane domain elicited greater target-dependent cytokine release and activation-induced cell death compared to CAR T-cells in which these elements

originated from CD8 α [14]. While *in vivo* potency was similar, use of a CD8 α hinge and transmembrane region may cause reduced cytokine release and limit toxicity in patients [14].

Costimulatory domains

Clinically evaluated second generation CARs utilize either a CD28 or 4-1BB costimulatory module and both have shown impressive outcomes [9, 15]. Initial pre-clinical comparisons of anti-CD19 CD28 ζ and 4-1BB ζ CARs demonstrated comparable anti-tumor activity, although cytokine release appears to be slower and of lower magnitude with 4-1BB ζ CARs [16, 17]. Many pre-clinical studies have employed high T-cell doses, leading to complete tumor eradication, meaning that subtle differences may remain undetected. By lowering T-cell doses, Zhao et al. demonstrated that anti-CD19 4-1BB ζ CAR T-cells mediate slower tumor elimination than CD28 ζ , but have greater persistence, thus resulting in similar overall therapeutic benefit [18].

Vector delivery systems

Most commonly, CAR-encoding genes are delivered to T-cells using γ -retroviral or lentiviral vectors [6]. Concerns regarding the potential risk of insertional mutagenesis have not been realized in practice as yet [19]. Alternative strategies include mRNA [20] or transposon/transposase plasmid electroporation [21]. Vector and promoter selection may have several consequences with regard to CAR T-cell efficacy and persistence. Retrovirally-transduced CARs may be subject to transcriptional repression, particularly during periods of quiescence. To mitigate this, Thomas et al. have shown that the insertion of a human β -interferon scaffold attachment region (SAR) into a γ -retroviral vector promoted sustained CAR transgene expression, when compared to non-SAR containing controls [22]. Ligand-independent CAR tonic signaling has been identified in a number of disparate settings

(typically using scFv-containing CARs) [23-26] and may arise as a consequence of high CAR surface expression. The latter is influenced in part by promoter strength and the type of vector used. Gomes-Silva et al. recently reported that 4-1BB-mediated ligand-independent tonic signaling may lead to enhanced apoptosis and poor ex vivo expansion due to a self-amplifying NF- κ B positive feedback loop, acting at the level of the γ -retroviral long terminal repeat (LTR) promoter [25]. These issues may be addressed by using self-inactivating (SIN) or non-integrating lentiviral vectors (NILVs) [27]. More recently, CARs have been introduced by precise gene editing using CRISPR/Cas9 [28] or engineered homing endonucleases [29]. Placement of the CAR gene within the TCR α constant (TRAC) locus has been shown to recapitulate endogenous TCR expression and recycling, thereby alleviating the deleterious effects of tonic signaling upon terminal differentiation, exhaustion and anti-tumor activity [28]. This approach also lends itself to the production of allogeneic “off the shelf” CAR T-cells by simultaneously knocking out functional TCR, thus minimizing the risk of graft versus host disease [30].

Next generation CARs

Considerable progress has been made in further engineering CAR T-cells to optimize in vivo performance and safety [31]. Various strategies have been deployed to enhance intrinsic CAR function [32]; lower the threshold for activation [33]; aid trafficking and intra-tumoral migration [34, 35]; engineer CAR temporo-spatial control [36, 37] and to enable the pharmacologically regulated elimination of these cells in the event of excessive toxicity [38]. To limit antigen escape and/or facilitate pattern recognition on tumor cells, CARs have been designed with dissociated endodomains placed *in trans* [39, 40], dual targeting domains (tandem CARs) [41] or logic circuits [42]. To enhance fitness of CAR T-cells, efforts have been made to optimize their differentiation [43] and metabolic capacity [44]. Alternatively,

potency of tumor attack may be enhanced by recruitment/ induction of innate and adaptive anti-tumor immune responses [45, 46]; resistance to intrinsic [34, 47, 48] or induced [49] immunosuppression in the tumor microenvironment (TME). Chimeric antigen receptors have also been designed to function in a more modular and customisable fashion [50] in anticipation of available “off the shelf” cellular therapeutics [51]. An overview of these approaches is summarized in Figure 2 while individual strategies are described in the sections that follow.

Dual antigen targeting

The emergence of acquired resistance to CAR T-cell immunotherapy through antigen loss [52] or lineage plasticity [53] had been predicted using several preclinical models. Immunotherapy of patients with B-cell malignancy has resulted in CD19⁺ clonal escape, mediated at least in part by alternative exon splicing [54]. In a mouse model of CAR-mediated antigen loss, dual expression of CARs targeting CD19 and CD123 led to superior activity against human B-ALL compared to single targeted CAR T-cells or the pooled combination of CD19 and CD123-directed T-cells. Likewise, the design of tandem CARs containing two or more extracellular binding moieties (e.g. targeting CD19 and CD20 [55] or CD19 and CD22 [56]) may recapitulate the advantages of using multiple CARs in a single CAR molecule.

Logic-gated CARs

The paucity of unique tumor-specific cell surface antigens means that substantial concerns remain regarding risk of on-target off-tumor toxicity. Logic-gated CARs utilizing Boolean operations such as AND, OR and NOT have been designed to enhance specificity and potentially reduce off-target effects [57]. Their development provides the rudimentary

tools for generating programmable CAR T-cells capable of sophisticated pattern recognition. OR gates may be enforced by using multiple CARs *in trans* or a single tandem CARs. Thus far, NOT gates have typically relied upon co-expressed inhibitory CARs (termed iCARs) containing an immune checkpoint-derived endodomain (e.g. from PD-1) [58]. However, differences in antigen density and/or the strength of positive and negative signaling may interfere with the function of iCARs *in vivo*. Designing an efficacious AND gate is somewhat more complex but various strategies have been described. Cordoba et al. have developed a strategy whereby an antigen targeting moiety is bound to a CD45 or CD148 phosphatase-derived transmembrane and endodomain [59]. These phosphatases typically play a role in regulating TCR-mediated cytolytic function and, owing to the large size of their ectodomain, they are sterically excluded from the immune synapse (as predicted by the kinetic segregation model) [60]. By contrast, unbound chimeric CD45 or CD148 may negatively regulate activation of a second CAR in a tonic fashion. Binding of target antigen sequesters the chimeric phosphatase, abrogating tonic CAR ITAM dephosphorylation and allowing the latter to induce T-cell activation following the binding of a second cognate ligand.

In an alternative strategy, Roybal et al. have designed a customizable logic-gated gene circuit system termed synNotch. To create synNotch, an extracellular targeting moiety is fused to a cleavable transmembrane domain (derived from the Notch receptor's core regulatory region) followed by an endodomain containing a synthetic transcription factor [42]. Upon target binding, the synNotch receptor is cleaved at the cell surface by metalloproteases and γ -secretase, releasing the synthetic transcription factor to induce orthogonal expression of a secondary CAR or iCAR. Potential limiting factors include the immunogenicity of using non-native or synthetic transcription factors, the relatively slow kinetics of the system due to its reliance on induced transcription and potential off-target effects due to synNotch CAR T-cells exiting the tumor following initial activation.

Another potential issue pertinent to AND-gated systems relates to selective antigen loss due to clonal evolution and tumor heterogeneity. Conceptually, this may be addressed by using multiple programmable CAR circuits that introduce fuzzy, conditional and/or non-binary (i.e. analog) outcomes. Illustrating this, split CARs may be employed whereby CD3 ζ and costimulatory domains are spatially separated *in trans* and bound to different targeting moieties [39].

Growth promoting and costimulatory receptors

A plethora of additional endogenous or synthetic chimeric receptor types have been introduced into CAR T-cells to optimize function in a paracrine fashion. Examples include chimeric cytokine receptors comprising an IL-4R α ectodomain and IL-2/15R β [61] or IL-7R α [62] endodomain. Both mediate enhanced ex vivo expansion and enrichment of CAR T-cells following exposure to IL-4. Alternatively, enforced overexpression of IL7R α may achieve IL-7-dependent increase in anti-tumor activity accompanied by Treg resistance [63]. Expression of CD40 ligand may be used to promote activation of professional antigen presenting cells [45]. A membrane-tethered chimeric IL-15/ IL-15 receptor α fusion molecule has been used to enhance T-cell persistence and memory stem-cell phenotype independent of CAR signaling [64]. Expression of 4-1BB ligand in CAR T-cells promotes enhanced juxtacrine activation of these T-cells and neighboring endogenous T-cells [18]. Finally, chimeric switch receptors may be employed that link the extracellular domain of an inhibitory immune checkpoint receptor with the intracellular signaling domain of a costimulatory receptor (e.g. PD-1/CD28 or PD-1/4-1BB) [65], thereby mitigating CAR T-cell exhaustion in the tumor microenvironment.

TRUCKs and armored CARs

“Armored” CARs have been engineered to constitutively produce cytokines that favor CAR T-cell expansion and effector function, accompanied by resistance to negative influences of the TME such as Tregs. T-cells that have been engineered to co-express a CAR and a cytokine (e.g. IL-12) that is produced in an NFAT-inducible fashion following activation have been termed “TRUCKs” [66]. Similarly, CD19-directed CARs engineered to secrete single chain IL-12 could safely eradicate established disease despite the absence of prior lymphodepleting conditioning [67]. Such an approach may simultaneously induce a bystander effect by re-programming tumor-resident myeloid cells to cross-prime endogenous HLA-restricted tumor-infiltrating lymphocytes (TILs) [68] or by inducing macrophages to target cancer cells that have evolved to downregulate antigen presentation [69]. CD19-specific CAR T-cells engineered to constitutively express the γ c-cytokines IL-2, IL-7, IL-15 or IL-21 all demonstrated enhanced anti-tumor efficacy in immune compromised mice, albeit to varying degrees and through singularly different mechanisms. Specifically, IL-7 and IL-21-transduced T-cells demonstrated greatest efficacy *in vivo*, while IL-21 and IL-15 promoted long-term CAR T-cell persistence, albeit with divergent effector memory and central memory phenotypes [70]. Separately, CD19-directed CAR T-cells have been engineered to constitutively express IL-15, improving antigen-driven expansion and efficacy while reducing exhaustion and apoptosis [71]. Indeed, local production of IL-15 was shown to elicit local CAR T-cell activation via activation of the phosphatidylinositol 3'-kinase/Akt pathway, resulting in upregulated expression of anti-apoptotic molecules such as Bcl-2 [72]. Contrasting with the “TRUCK” approach, Koneru et al. designed a MUC16-specific CAR in which the IL-12 gene was placed downstream of an internal ribosome entry site (IRES) element. This approach resulted in the production of lower levels of IL-12 compared to CARs expressing NFAT IL-12 and was found to cause less toxicity in mice [73] [74]. Based on the evidence of efficacy against human ovarian tumours in mouse models, this armored IL-12

secreting CAR is now undergoing Phase I clinical testing in patients with epithelial ovarian cancer (NCT02498912). Comparably, GD2-redirected CAR T-cells engineered to express a constitutively active form of Akt were found to exhibit improved function, survival and intrinsic resistance to immunosuppression due to higher sustained levels of NF- κ B and anti-apoptotic genes, such as Bcl-2, Mcl-1 and Bcl-xL [47]. These benefits, however, may be offset by the emergence of a more terminally differentiated effector memory phenotype and sub-optimal metabolic capacity [75]. Another approach to generate armored CAR T-cells is through the co-expression of CD40L. Curran et al. generated a CD19-directed CAR that constitutively co-expressed CD40L and which mediated enhanced anti-tumor activity in vitro. Enhanced survival benefit was observed when DoHH2 tumor bearing mice were treated with CD40L-containing armored CAR T-cells, compared with matched control second generation CAR T-cells. This approach alters the tumor microenvironment in a favorable manner, thereby enhancing anti-tumor activity [45].

Strategies to enhance CAR T-cell intra-tumoral migration

Inadequate CAR T-cell trafficking may compromise efficacy, particularly against solid tumors. Several strategies have been proposed to address physical and/or molecular barriers to CAR T-cell entry. The most straightforward of these entails direct intra-tumoral or intra-cavitary CAR T-cell delivery [76]. This approach is supported by evidence that CAR T-cells largely remain within the tumor after direct delivery [77]. Alternatively, chemokine/chemokine receptor circuits may be harnessed to address this limitation. For example, expression of the CXCR3 chemokine receptor may be enhanced by blocking protein kinase A localization [34]. Since CAR T-cells are genetically engineered, vectors may alternatively be used to co-deliver chemokine receptors that are matched to tumor-specific chemokines [35, 78]. In a related approach, tumor-tropic oncolytic viruses may be armed with chemokines

and/or cytokines and administered prior to CAR T-cell immunotherapy [79]. Penetration of CAR T-cells within solid tumors is also recognized to be an important hurdle, particularly given the high interstitial pressures that pertain within this environment. To address this, CAR T-cells may be engineered to secrete heparanase to disrupt tumor-associated extracellular matrix [80]. In addition, tumor cell surface adhesion molecules may be upregulated to aid CAR T-cell functionality [81].

Enhancing CAR metabolism

The TME is often inhospitable to T-cells due to local hypoxia, low pH, high lactate, the absence of critical amino acids such as tryptophan or arginine and the presence of immunosuppressive mediators such as kynurenine, prostaglandin E₂ and adenosine. Various strategies have been proposed to optimize CAR T-cell metabolism in the face of these obstacles. One approach entails the overexpression of peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 α), which leads to enhanced oxidative phosphorylation and mitochondrial biogenesis [75]. Since Akt is a negative regulator of PGC-1 α , inhibition of Akt represents an alternative strategy to achieve this goal. Akt been shown to phosphorylate and sequester FOXO in the cytoplasm, thereby inhibiting the transcription of FOXO-inducible molecules associated with central memory or stem memory phenotype. Pharmacological modulation of this pathway can be achieved using Akt or PI3K δ inhibitors during *ex vivo* expansion of CAR T-cells, leading to enhanced expression of memory markers and superior anti-tumor activity [82]. Similar effects have been reported in response to enhanced canonical Wnt/ β -catenin signaling, which has been shown to confer a stem memory T-cell phenotype [83]. Memory T-cell differentiation may also be favored by inhibition of glycolysis (e.g. using the glucose analog 2-deoxyglucose [84]), enforced fatty acid oxidation (e.g. by over-expressing carnitine palmitoyltransferase 1a [85]) or by re-

programming of mitochondrial function to achieve a T-cell metabolic phenotype in which catabolic pathways (e.g. oxidative phosphorylation and fatty acid oxidation) dominate over anabolic processes (e.g. glycolysis) [86].

Engineering safety in CAR T-cells

The use of CAR T-cell immunotherapy in the clinic has elicited concerns regarding on-target and off-target toxic effects. Particularly severe toxicities include cytokine release syndrome (CRS), macrophage activation syndrome (MAS) and neurotoxicity, all of which may result in fatal complications [87]. Adoptive cell transfer of 10^{10} HER2-redirected CAR T-cells in a patient with HER2⁺ metastatic colorectal cancer resulted in fatal multi-organ failure due to the binding of low-level antigen on pulmonary epithelium and/ or microvasculature [8]. Temporospatial control of CAR T-cells may help to manage these risks and can be achieved by engineering on- or off-switches or inducible suicide systems. Herpes simplex virus thymidine kinase (HSV-TK)-engineered T-cells are amenable to pharmacologic clearance by administration of ganciclovir. This approach has been clinically validated in patients undergoing allogeneic hematopoietic stem cell transplantation. However, the HSV-TK gene product is highly immunogenic and thus is liable to promote immune-mediated clearance of engineered cells [88]. Prototypic tetracycline (tet)-on and tet-off systems subjugate target gene transcription to the presence or absence of this antibiotic, or one of its derivatives. Sakemura et al. have expressed a second generation CD19-specific CAR using a third generation tet-on vector, demonstrating loss of CAR expression and function in the absence of doxycycline [89]. Alternatively, heterodimerizing agents such as AP21967 or gibberellin have been utilized in a split CAR construct, whereby the extracellular antigen-binding moiety is linked to an intracellular heterodimerizing module +/- a costimulatory domain. Following the delivery of AP21967 or gibberellin, this module

heterodimerizes with a second DAP10-linked molecule containing a CD3 ζ domain, alone or with a further costimulatory domain [37]. Separately, a system comprising a myristoylated membrane-tethered chimeric MyD88/CD40 endodomain (GoCART) provides signal 2 for optimal CAR function only in the presence of the chemical inducer of dimerization (CID), rimiducid [36].

The insertion of a suicide gene cassette comprising inducible caspase-9 (iCas9) has also been utilized to enhance CAR safety. Delivery of CIDs (AP1903 or AP20187) induce homodimerization of a chimeric molecule comprising caspase-9 and a drug-binding domain, leading to the activation of downstream pro-apoptotic molecules and CAR T-cell death [38]. Alternatively, the co-expression of a truncated cell surface molecule such as epidermal growth factor receptor or CD20 that is the target of a clinical grade monoclonal antibody (such as cetuximab or rituximab) allows for selective elimination of CAR T-cells via antibody- and/ or complement-dependent cell-mediated cytotoxicity [30]. Such systems rely upon uniform expression of the safety switch following T-cell transduction to avoid CAR T-cell escape.

In a distinct approach to restrict CAR T-cell activation to the TME, transcription of the CAR-encoding gene has been coupled to a hypoxia-sensitive subdomain of hypoxia-inducible factor-1 alpha (HIF-1 α), leading to enhanced CAR cell surface expression in the context of tumor hypoxia [90]. Nonetheless concerns remain about leakiness of gene expression using this approach. Likewise, antigen-specific CD8⁺ T-cells expanded in hypoxic conditions prior to adoptive cell transfer have been shown to exhibit greater intrinsic effector function due to higher concentrations of granzyme B in their cytolytic granules [91].

Universal modular CAR designs

Various customizable CAR platforms have been designed to reduce the financial and human costs associated with the development of individual antigen-targeting CARs. Such approaches are particularly welcome in anticipation of the development of “off the shelf” allogeneic CAR products. Examples include Fc-specific CARs [92] or orthogonal systems whereby a single CAR is designed to target a non-human proteome-derived peptide bound to antibody, Fab or scFv targeting modules [50, 93]. Antigen escape may therefore be addressed by using multiple exchangeable antibody-bound targeting modules, but the system is reliant upon adequate penetration of the TME and sufficient tumor cell surface binding by the antibody-conjugated targeting module.

Summary

Clinical studies have shown impressive results of CAR T-cell therapy and have revolutionized the treatment landscape for patients with B-cell malignancies. Further pre-clinical development and improvements will be crucial to translate these results in other malignancies and also address issues that have arisen post-treatment. Modifications of CAR scFvs, hinge and costimulatory domains have the potential to address many of the current limitations of CARs such as persistence whereas the generation of armored CARs or ‘TRUCKS’, to achieve the release of cytokines will enhance their expansion and survival. Other augmentations that will improve tumor specificity of CAR T-cells include the targeting of multiple antigens, which could reduce the chance of antigen escape and also enhance safety. Additional engineering of CARs to include suicide genes or iCARs are other possible ways to achieve safety and address toxicity, which remains an issue in the clinic. Ultimately, pre-clinical research will help to illuminate which strategy or optimization will be best to enable the use of CAR T-cells safely and widely.

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Conflict of Interest

JM is chief scientific officer of Leucid Bio. The other authors have no conflict of interest to declare.

Practice Points

- CAR T-cell therapies have yielded unprecedented response rates and survival outcomes in patients with chemotherapy-resistant and refractory B-cell malignancies.
- Preclinical evaluation of CD28 versus 4-1BB-containing second generation CD19-directed CAR T-cells have revealed differences in cytokine release, effector function and persistence.
- Reducing risk of toxicities such as cytokine-release syndrome and cerebral oedema is essential for improving CAR T-cell therapy.
- Next generation CAR T-cells currently in clinical development have been further engineered to enhance polyfunctionality and safety, as such it is likely that CAR T-cell therapy will become a standard treatment option for patients in the future.

Research Agenda

- CD19-directed CAR T-cells may fail to induce long-term remissions due to the emergence of antigen loss or impaired CAR T-cell persistence. Further research is required to address both issues.
- In addition, the development of safe and effective CAR T-cell therapies for patients with non-B-cell malignancies and solid tumors remains compromised by a paucity of targetable tumour-associated antigens.
- Strategies designed to control CAR T-cell functionality spatially, temporally and in a modular fashion may also improve safety and efficacy but require clinical evaluation.

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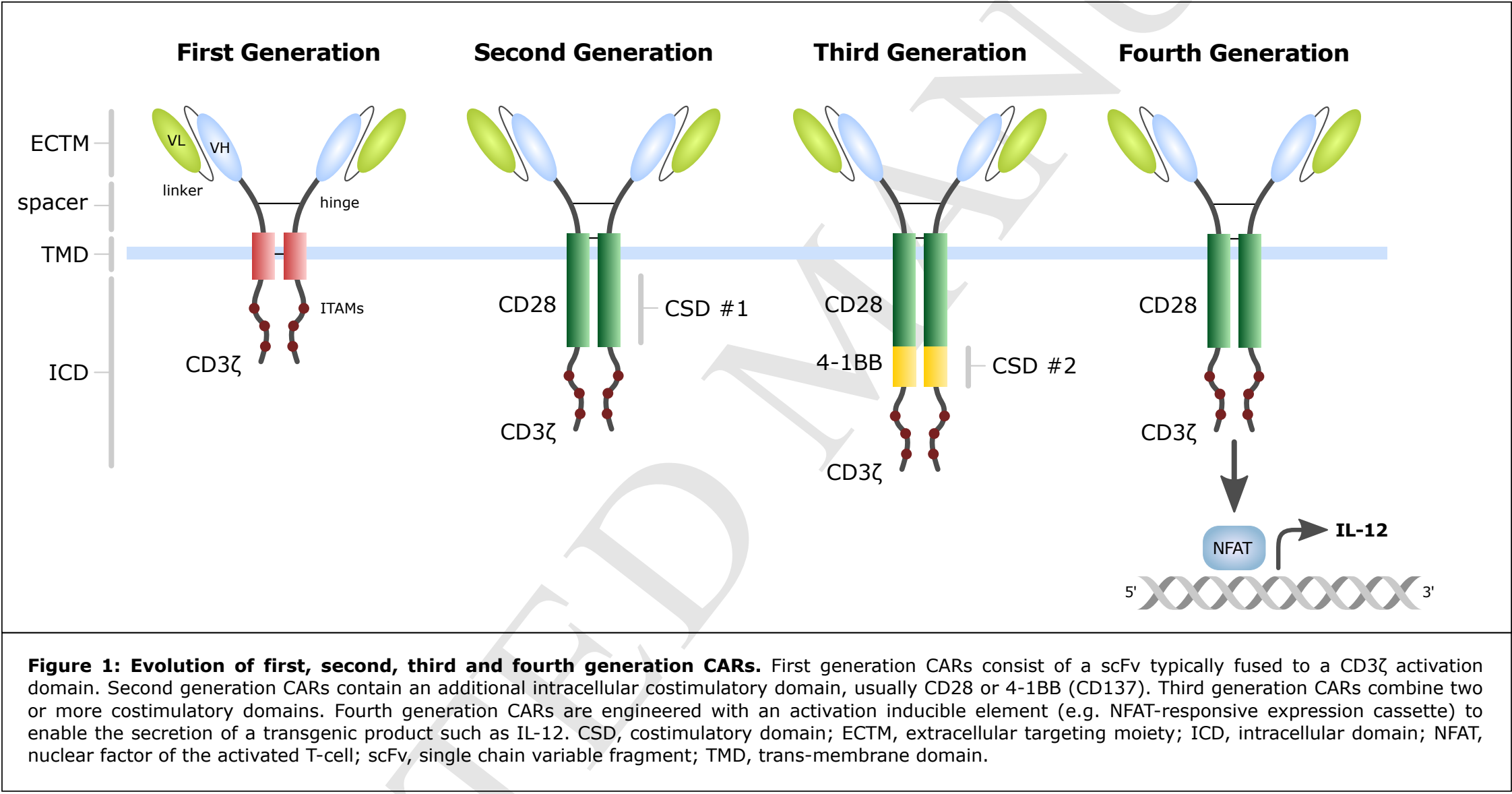
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Figure legends:

Figure 1: Evolution of first, second, third and fourth generation CARs. First generation CARs consist of a scFv typically fused to a CD3 ζ activation domain. Second generation CARs contain an additional intracellular costimulatory domain, usually CD28 or 4-1BB (CD137). Third generation CARs combine two or more costimulatory domains. Fourth generation CARs are engineered with an activation inducible element (e.g. NFAT-responsive expression cassette) to enable the secretion of a transgenic product such as IL-12. CSD, costimulatory domain; ECTM, extracellular targeting moiety; ICD, intracellular domain; NFAT, nuclear factor of the activated T-cell; scFv, single chain variable fragment; TMD, trans-membrane domain.

Figure 2: CAR T-cell engineering for enhanced efficacy & safety. (A) CAR design: second and third generation CARs incorporate a variety of costimulatory intracellular domains such as CD28, 4-1BB, OX40, ICOS, CD27 or DAP10; these may be further engineered to alter the functional characteristics of the CAR T-cells (e.g. mutation of the Lck-binding site in CD28 may reduce IL-2 production & minimize Treg expansion); the extracellular scFv may be substituted with targeting moieties derived from endogenous molecules (e.g. the pan-ErbB ligand, T1E [94] or NKG2D [95]), single domain nanobodies or monomeric fibronectin-based domains; adjustments to the spacer and/or transmembrane domain may also impact upon efficacy. (B) Expression of surface costimulatory molecules: examples include chimeric cytokine receptors (e.g. IL-4 α /IL-2R β), 4-1BBL, CD40L, PD-1-based costimulatory switch receptors or a membrane tethered chimeric IL-15 fusion molecule. (C) Expression of matched chemokine receptors for enhanced intra-tumoral trafficking. (D) Constitutive or induced secretion of cytokines, collagenases or molecules targeting TME immunosuppression (e.g. anti-PD-1 monoclonal antibodies). (E) Optimizing CAR T-cell metabolic capacity and phenotype e.g. by overexpressing PGC-1 α , modulating the PI3K-Akt or canonical Wnt/ β -catenin pathways. (F) Inserting safety systems to address toxicity (e.g. HSV-TK, iCas9, truncated EGFR or CD20 targetable with mAbs); engineering spatiotemporal control by using chemical inducers of dimerization acting at the level of a split CAR or a separate costimulatory module or by regulating gene transcription using the synNotch system. (G) Optimizing CAR expression and recycling by utilizing CRISPR/Cas9 or homing endonucleases to position the CAR within the TRAC locus; utilising self-inactivating or non-integrative viral vectors, mRNA or transposon/transposase systems.



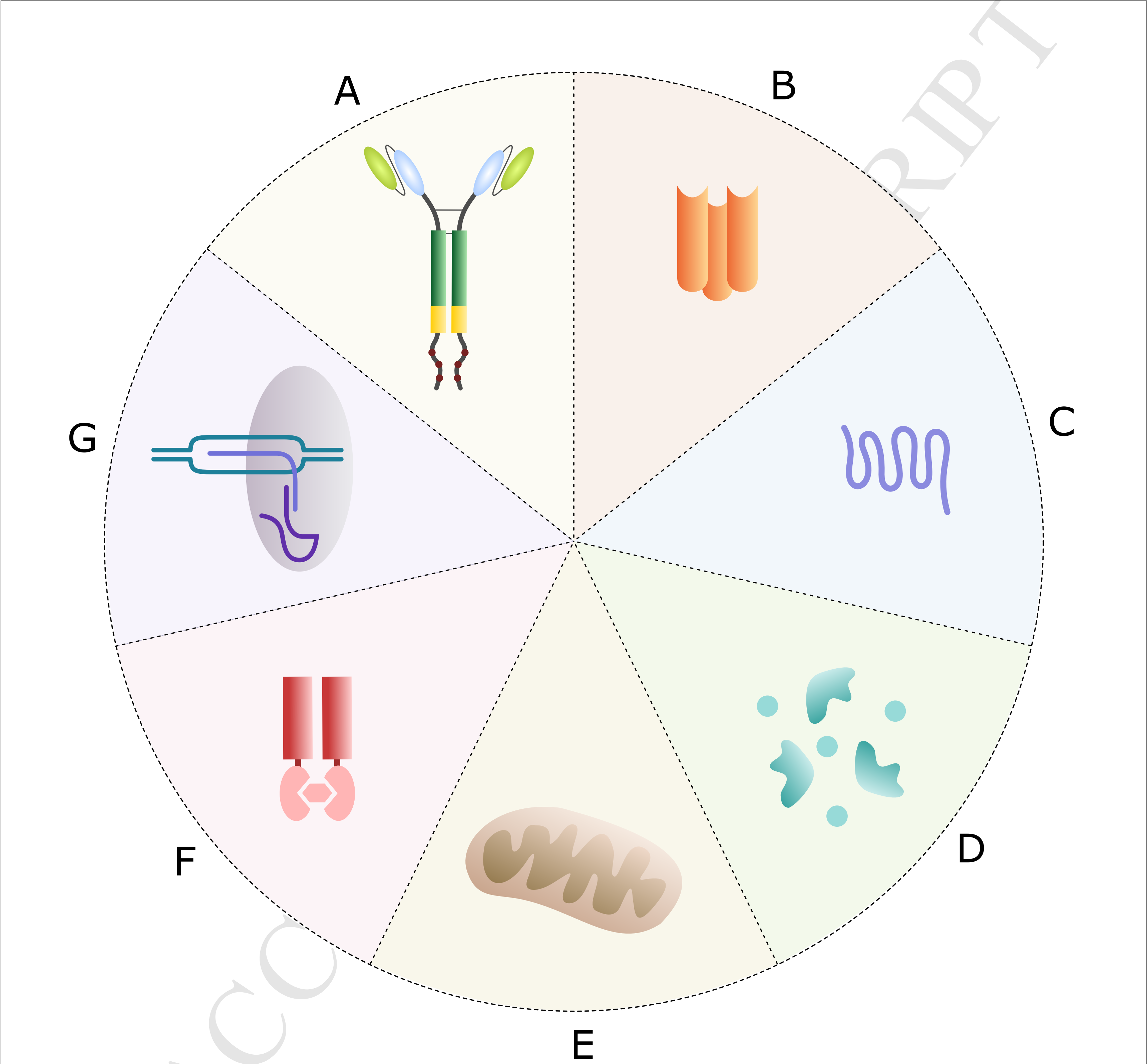


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